American Journal of Medical Genetics 70:299–302 (1997)

Novel Point Mutation in the Uroporphyrinogen III Synthase Gene Causes Congenital Erythropoietic Porphyria of a Japanese Family

Noboru Takamura,[1] Isabelle Hombrados,[3] Ken Tanigawa,[1] Hiroyuki Namba,[1] Yuji Nagayama,[2] Hubert de Verneuil,[3] and Shunichi Yamashita[1]*

[1]*Department of Preventive Medicine, Atomic Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan*
[2]*Department of Pharmacology 1, Nagasaki University School of Medicine, Nagasaki, Japan*
[3]*Laboratorie de Pathologie Moléculaire et Thérapie Génique, Université de Bordeaux II-146, Bordeaux Cedex, France*

The molecular basis of the uroporphyrinogen III synthase (UROIIIS) deficiency was investigated in a member of a Japanese family. This defect in heme biosynthesis is responsible for a rare autosomal recessive disease: congenital erythropoietic porphyria (CEP) or Günther's disease. The patient was homozygous for a novel missense mutation: a G to T transition of nucleotide 7 that predicted a valine to phenylalanine substitution at residue 3 (V3F). The parents were heterozygous for the same mutation. The loss of UROIIIS activity was verified by an in vitro assay system. The corresponding mutated protein was expressed in *Escherichia coli* and no residual activity was observed. Further studies are needed to determine whether the mutations of the UROIIIS gene (*UROS*) have a specific profile in Japan compared to European or American countries. Am. J. Med. Genet. 70:299–302, 1997.

KEY WORDS: uroporphyrinogen III synthase (UROIIIS); congenital erythropoietic porphyria (CEP)

INTRODUCTION

Porphyrias are a group of inherited disorders caused by specific defects along the heme biosynthetic pathway [Kappas et al., 1989]. Congenital erythropoietic porphyria (CEP), or Günther's disease, is a rare disorder inherited as an autosomal recessive mode. The incidence of this disease is not known, but is exceedingly low; less than 200 cases were reported [Moore et al., 1987; Kappas et al., 1989]. CEP is characterized by severe cutaneous photosensitivity, chronic hemolysis, and massive porphyrinuria, resulting from the accumulation in the bone marrow, peripheral blood, and other organs of large amounts of predominantly type I porphyrins, which are not substrates for heme synthesis [Romeo and Levin, 1969; Deybach et al., 1980; Moore et al., 1987; Kappas et al., 1995]. A characteristic abnormality of the disease is an 80–98% reduction in the activity of uroporphyrinogen III synthase (UROIIIS), the fourth enzyme of the heme biosynthetic pathway (EC. 4.2.1.75) [Romeo and Levin, 1969; Deybach et al., 1981].

Since the cDNA of the UROIIIS has been cloned and sequenced by Tsai and co-workers [1988], attention has focused on the molecular defects responsible for CEP. To date, 17 different mutations of the UROIIIS gene (*UROS*) have been described [Deybach et al., 1990; Boulechfar et al., 1992; Warner et al., 1992; Bensidhoum et al., 1995; Xu et al., 1995; Tanigawa et al., 1996] and a single mutation, C73R, is present in 50% of the disease alleles in the Europeans and is associated with a severe phenotype. In Japan, we have recently reviewed 33 cases of CEP and found that only 4 cases are analyzed at the molecular level [Xu et al., 1995; Tanigawa et al., 1996]. Identification of mutations of *UROS* is important for genetic counseling and prenatal diagnosis of affected families [Ged et al., 1996].

In this study, we describe the identification of a novel *UROS* mutation in a Japanese family and we show that the base change is disease-causing mutation, through heterologous expression in *E. coli*.

MATERIALS AND METHODS

Case Report

We studied a CEP man, who lived in the Fukushima prefecture of Japan and his family members, after obtaining informed consents. The family pedigree is shown in Figure 1. The patient, a 34-year-old male, had

*Correspondence to: Dr. Shunichi Yamashita, Nagasaki University School of Medicine, Sakamoto 1-12-4, Nagasaki 852, Japan.

Received 17 July 1996; Accepted 1 October 1996

classical manifestations of CEP, starting shortly after birth. High performance liquid chromatography of urine also showed a porphyrin excretion profile predominantly of Type I isomers in the patient. UROIIIS enzyme activity was not measurable (<2% of the normal value) in the erythrocytes of the patient. Both parents have 50% residual activity (Fig. 1).

UROIIIS Assay in Erythrocytes

UROIIIS activity was determined by the enzyme coupled assay described previously with modifications [Deybach et al., 1981]. Whole blood (10 μl) were sonicated on ice in 250 μl of 100 mM Tris-Cl, pH 8.0, containing 1 mM EDTA and 1% Tween. Aliquots of the 12,000g supernatant were incubated with porphobilinogen and the amount of porphobilinogen deaminase (or hydroxymethylbilane synthase) catalyzing the formation of 200 pmoles of uroporphyrinogen in the assay conditions. Partially purified porphobilinogen deaminase was prepared by heating a human hemolysate at 60°C for 90 minutes. The incubation mixture for the UROIIIS assay contains 25 nmol porphobilinogen, 250 nmol dithiotreitol, 75 μl porphobilinogen deaminase (corresponding to the formation of 200 pmoles of uroporphyrinogen), 5 to 50 μl of hemolysates, and 2.5 μmol of Tris-Cl, pH 8.0, in a total volume of 250 μl. Incubation was carried out at 37°C for 15 minutes in the dark. The reaction was stopped by addition of 25 μl of 50% trichloroacetic acid. The oxidized uroporphyrin isomers were separated by C18 reversed phase high performance liquid chromatography using 15% acetonitrile in 1 M ammonium acetate buffer, pH 5.2, and quantitated using a spectrofluorimeter as a detector. Porphyrins were detected by their fluorescent emission at 618 nm after excitation at 404 nm. One unit of UROIIIS activity was defined as the amount of enzyme which formed 1 nanomole of uroporphyrinogen III per hour at 37°C. Enzyme assay in the bacterial lysate was measured as previously described [Boulechfar et al., 1992; Bensidhoum et al., 1995].

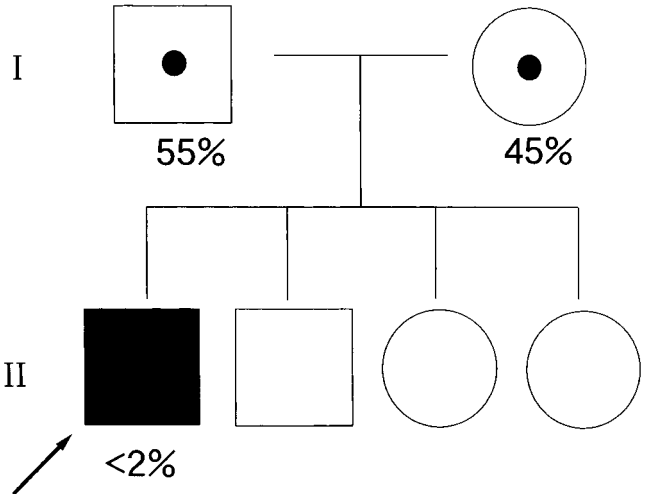

Fig. 1. Pedigree of the Japanese family. Percentage is UROIIIS erythrocyte activity for the patient and parents. Enzyme activity in normal erythrocytes is 5.36+1.34 (SD) U/mg protein.

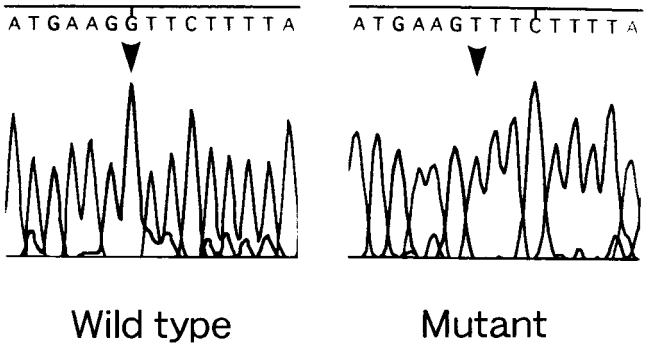

Fig. 2. Fluorescence based dye-primer sequencing was performed on the last exonic fragment of the gene, amplified by PCR genomic DNA. Patient is homozygous for a G to T change at codon 7 (V3F, **left panel**) as well as her mother, while her father has a normal allele (**right panel**).

Genomic DNA Extraction and Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from peripheral blood leukocytes using DNA Extractor WB Kit (Wako Pure Chemical Industries, Japan) and each exon including its intron-exon boundaries was amplified by PCR using primer sets previously described [Warner et al., 1992; Bensidhoum et al., 1995; Xu et al., 1995]. Amplified PCR products were purified on 1% agarose gels by QIAX kit (QIAGEN, Germany).

Subcloning and DNA Sequencing

For the sequencing, purified PCR products were ligated to the pCR™II vector (TA cloning kit, Invitrogen Corporation, La Jolla, CA) and chimeric vectors were used to transform competent *E. coli* cells. Fifteen colonies of each exon were picked up and after small scale preparation of plasmid DNA, the products were purified by FlexiPrep Kit (Pharmacia Biotech, Sweden), subjected to a fluorescence-based dye primer sequence reaction with a PRISM™ Ready Reaction dye Primer Cycle Sequencing Kit (Perkin-Elmer, Oak Brook, IL), and then sequenced with a Thermo Sequenase Core Sequencing Kit (Amersham International, England) and a Hitachi SQ-5500DNA Sequencer (Hitachi Electronics Engineering Co., Japan).

Construction and Functional Expression of the Mutant UROIIIS cDNA

The mutant UROS-cDNA was constructed with Ex-Site PCR-Based Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacture's instruction. The wt-UROS-cDNA [Boulechfar et al., 1992] in the bacterial expression vector pKK223-3 (Pharmacia Biotech, Sweden) was used as a template. The oligonucleotide primers used were 5′-ATAATGAAG*T*TTCTTTTACTG-3′ (sense, nucleotide -3 to +18) and 5′-TGCCTGGC*AGGCCT*TATAGGG-3′ (antisense, -4 to -24), where the mutated nucleotide is underlined, and the italics is a *Stu*I restriction site that allows selecting the clones harbouring the mutation. The mutated and adjacent regions were confirmed by sequencing. The resultant plasmid was designated pKK-UROIIIS-V3F. *E. coli* cells were transformed with

TABLE I. Specific Activity of Normal and Mutated UROIIIS Proteins*

Plasmid	Specific activity (U/mg protein) Mean	SD	Number of assays	Residual activity
pKK	0.35	±0.1	5	—
pKK-UROIIIS (wt)	143	±35	4	100
pKK-UROIIIS (V3F)	0.37	±0.9	3	<2

*Results of specific activity (SA) are the mean of three to five experiments. Residual activity was determined by dividing 100 × [SA – SA(pKK)] by [SA(pKK – UROSIIIS) – SA(pKK)].

pKK-UROIIIS-V3F or pKK-wt-UROIIIS and grown at 37°C. The protein expression was then induced by growing the cells in the presence of IPTG as previously described [Boulechfar et al., 1992; Bensidhoum et al., 1995]. UROIIIS specific activity (SA) is expressed as the relative amount of UROIIIS (nmoles) formed in 1 hour/mg of protein. Results are the mean of three to five experiments. Residual activity (as a percentage of the normal UROIIIS specific activity) was determined by dividing 100 × [SA-SA(pKK)] by [SA (pKKUROIIIS)-SA(pKK)] where SA(pKK) and SA(pKKUROIIIS) are the specific activities of the lysate (empty plasmid) and of the normal UROIIIS protein respectively.

RESULTS

The coding region of *UROS* had a normal size in the patient. In the patient, a single mutation was found at a homozygous state: a G to T change at nucleotide 7 leading to a valine to phenylalanine substitution at codon 3 (V3F), as shown in Figure 2. Furthermore, in both parents of this patient, the same mutation was found at the heterozygous state. This is not surprising since they were suspected to be a strong consanguinity based on the same geographic area in Japan, the Fukushima Prefecture.

In order to demonstrate that this base change is a disease-causing mutation, the corresponding mutated protein was expressed in *E. coli*. The activity of UROIIIS was measured in the bacterial lysate as previously described [Boulechfar et al., 1992; Bensidhoum et al., 1995]. No residual activity was observed in the mutated construct as compared to the normal pKK-UROIIIS (<2% of normal activity) (Table I).

DISCUSSION

To date, 17 different mutations in UROIIIS have been described in CEP patients: L4F, Y19C, T53M, T62A, A66V, C73R, V82F, V99A, A104V, 633insA, G225S, T228M, Q249X, IVS2^{+1}, IVS9$^{\Delta A+4}$, 148del98, and 60ins80A [Deybach et al., 1990; Boulechfar et al., 1992; Warner et al., 1992; Bensidhoum et al., 1995; Xu et al., 1995; Tanigawa et al., 1996]. A single mutation, C73R, has been frequently reported in European and American countries [Deybach et al., 1990; Boulechfar et al., 1992; Warner et al., 1992; Verstraeten et al., 1993; Bensidhoum et al., 1995; Xu et al., 1995; Ged et al., 1996; Tanigawa et al., 1996]. In Asian countries, such as Japan, the mutation C73R has not yet been observed. Recently, three mutations (T62A, T228M, and Q249X) were reported in Japanese patients [Xu et al., 1995; Tanigawa et al., 1996]. T62A has been observed only once in all CEP patients, and a patient who showed the mutation Q249X [Tanigawa et al., 1996], also present in our pedigree, may not live in the Kyushu Island in Japan.

V3F lesion did not occur at a CpG dinucleotide, a mutation hot spot due to deamination of metylcytosine to thymidine. Furthermore, the mutation V3F substituted isofunctional residues, but it had no residual activity in the bacterial expression system. The corresponding amino acid may play an important role in the function of the enzyme.

The epidemiological and clinical data of all porphyrias in Japan have been summarized [Tanigawa et al., 1995]. It is noticeable that 33 of 200 CEP cases have been reported in Japan. Further studies in Japanese CEP patients are currently performed to determine whether *UROS* mutations have a specific profile in Japan compared to European or American countries. Finally, same as other genetic disorders, a screening of CEP families with mutations at the DNA level will benefit to genetic counseling and prenatal diagnosis. HLA-identical allogenic bone-marrow transplantation can be proposed to severely affected patients [Kauffman et al., 1991]. Recent studies indicate the potential usefulness of somatic gene therapy in the future for this disease [Moreau-Gaudry et al., 1995].

ACKNOWLEDGMENTS

We cordially thank the CEP family who took part in this study.

REFERENCES

Bensidhoum M, Ged C, Hombrados I, Moreau-Gaudry F, Hift RS., Meissner P, Sturrock ED, de Verneuil H (1995): Identification of two new mutations in congenital erythropoietic porphyria. Eur J Hum Genet 3:102–107.

Boulechfar S, Da Silva V, Deybach JC, Nordmann Y, Grandchamp B, de Verneuil H (1992): Heterogeneity of mutations in the uroporphyrinogen III synthase gene in congenital erythropoietic porphyria. Hum Genet 88:320–324.

Deybach JC, Grandchamp B, Grelier M, Nordmann Y, Boue J, Boue A, de Berrianger P (1980): Prenatal exclusion of congenital erythropoietic porphyria (Günther's disease). Hum Genet 53:217–221.

Deybach JC, de Verneuil H, Phung N, Nordmann Y, Puissant A, Boffety B (1981): Congenital erythropoietic porphyria (Günther's disease): Enzymatic studies on two cases of late onset. J Lab Clin Med 97:551–558.

Deybach JC, de Verneuil H, Boulechfar S, Grandchamp B, Nordmann Y (1990): Point mutations in uroporphyrinogen III synthase gene in congenital erythropoietic porphyria. Blood 74:1763–1765.

Ged C, Moreau-Gaudry F, Taine L, Hombrados I, Calvas P, Colombies P,

Verneuil H (1996): Prenatal diagnosis in congenital erythropoietic porphyria. Prenat Diag 16:83–86.

Kappas A, Sassa S, Galbraith RA, Nordmann Y (1989): The porphyrias. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds) : "The Metabolic Basis of Inherited Disease." New York: Mac Graw-Hill, pp 1305–1365.

Kauffman L, Evans DI, Stevens RF, Weinkove C (1991): Bone-marrow transplantation for congenital erythropoietic porphyria. Lancet 337: 1510–1511.

Moore MR, McColl KL, Rimington C (1987): "Disorders of Porphyrin Metabolism." New York: Plenum Publishing, pp 213–226.

Moreau-Gaudry F, Ged C, Barbot C, Mazurier F, Boiron JM, Bensidhoum M, Reiffers J, de Verneuil H (1995): Metabolic correction of congenital erythropoietic porphyria by retrovirus-mediated gene transfer into EBV-transformed cell line. Blood 85:1449–1453.

Romeo G, Levin EY (1969): Uroporphyrinogen III cosynthase in human congenital erythropoietic porphyria. Proc Natl Acad Sci USA 63:856–863.

Tanigawa K, Takamura N, Yamashita S (1995): Congenital erythropoietic porphyria. Nippon Rinsho 110:1422–1426 (in Japanese).

Tanigawa K, Bensidhoum M, Takamura N, Namba H, Yamashita Y, de Verneuil H, Ged C (1996): A novel point mutation in congenital erythropoietic porphyria in two members of a Japanese family. Hum Genet 97:557–560.

Tsai SF, Bishop DF, Desnick RJ (1988): Human uroporphyrinogen III synthase: molecular cloning, nucleotide sequence and expression of a full-length cDNA. Proc Natl Acad Sci USA 85:7049–7053.

Verstraeten L, Rogemorter NV, Pardou A, de Verneuil H, Da Silva V, Rodesch F, Vermeylen D, Donner C, Noel JC, Nordmann Y, Hassoun A (1993): Biochemical diagnosis of a fetal case of Günther's disease in a new born with hydrops foetalis. Eur J Clin Chem Clin Biochem 31: 121–128.

Warner CA, Yoo HW, Roberts AG, Desnick RJ (1992): Congenital erythropoietic porphyria: Identification and expression of exon in mutations in the uroporphyrinogen III synthase gene. J Clin Invest 89:693–700.

Xu W, Warner CA, Desnick RJ (1995): Congenital erythropoietic porphyria: identification and expression of 10 mutations in the uroporphyrinogen III synthase gene. J Clin Invest 95:905–912.